**Supplementary Data**

# Identification of PCTA, a TGIF antagonist that promotes PML function in TGF-β signaling

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## Supplementary Materials and Methods

### Expression vectors

Expression vectors for $ARE_3$-Lux, Flag-Smad2, Flag-Smad3, Flag-Smad2.3SA HA-TβRI, HA-TβRI.act, Flag-TβRI, Flag-TβRI.act, Flag-TβRImL45, Flag-TβRImL45.act, Myc-cPML, Myc-Smad2, HA-Smad4, Myc-FAST1, HA-TGIF, Myc-TGIF, Myc-TGIF(1-164), Myc-TGIF(50-272) and Myc-TGIF(108-272) were previously described (Prunier et al., 2001; Seo et al., 2004; Seo et al., 2006). For the expression vector encoding Flag-cPML, cPML was amplified by PCR from pcDNA4-His-cPML (a gift from Dr. P. Pandolfi) and subcloned into pCMV10.3xFlag. For PCTA constructs, sequences for restriction enzymes were introduced into pEFplink2-PCTA (a gift from Dr. S. Goodbourn) by the Quick-Change Site-Directed Mutagenesis Kit (Stratagen) and PCTA cDNAs were digested and subcloned into pcDNA3-6xMyc, pCMV5-2xHA, pCMV10.3xFlag, pcDNA-3-HisC, or pcDNA5/TO. A similar strategy was used to generate the mutant form HA-PCTA.C503A/C506A (referred to as HA-PCTA.CA). For the expression vector encoding shPCTA, oligonucleotides containing sequences from human PCTA were inserted into the expression vector pBLOCK-iT™ according to the manufacturer's instructions (Invitrogen).

### Antibodies

For immunoprecipitation, immunoblotting or immunofluorescence the following antibodies were used: anti-Flag M2 (Sigma), anti-HA (Roche), anti-pSmad2 (UBI), anti-Smad2 (Zymed), anti-ADAM12, anti-p21Cip1, anti-c-Jun (Calbiochem), anti-c-Myc 9E10, anti-pSmad3, anti-SARA, anti-JNK, anti-TGIF, anti-LaminB, anti-Tubulin, anti-Smad4 and anti-PML (Santa Cruz Biotechnology).

### Cell lines and culture

HepG2, CaCO2, Mv1Lu, HaCat, Mv1Lu, 293, wild-type MEFs (for JNK1/JNK2, c-Jun or PML), PML-/- MEFs, c-Jun-/- MEFs, JNK1-/-JNK2-/- MEFs and MDCK cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal calf serum (FCS) and 5 mM glutamine. MDCK-pcDNA3, MDCK-TGIF, MDCK-Smad7 and MDCK-shPCTA cells were maintained in growth medium containing

neomycin. MDCKpSUPER, and MDCKpSUPER-TGIF were maintained in hygromycin-containing growth medium. MDCK-TR and MDCK-TR-PCTA were maintained in growth medium containing blastidin and hygromycin.

## Supplementary Figures

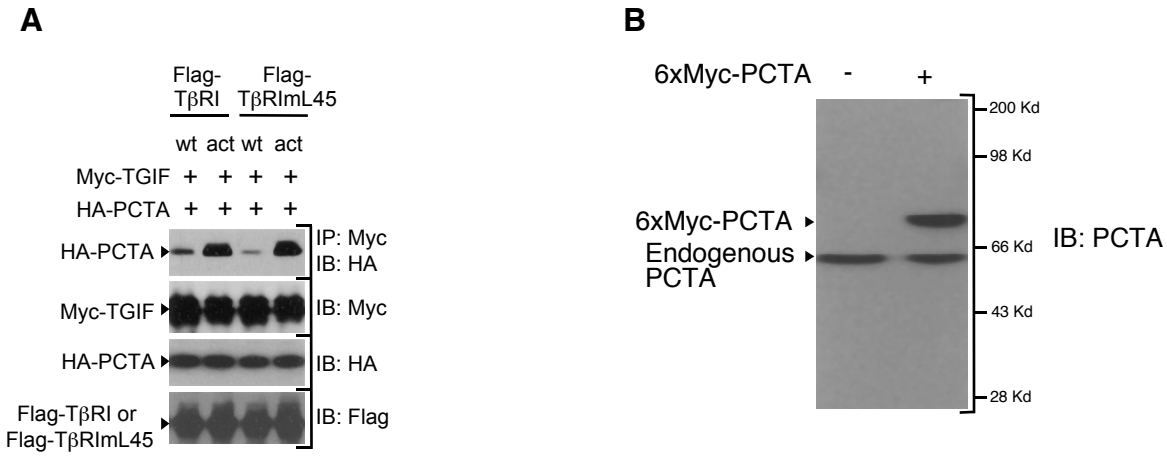


**Figure S1**
(**A**) TGF-β induces the assembly of PCTA/TGIF complex independently of activation of Smad signaling. 293 cells were transfected with the indicated combinations of HA-PCTA, Myc-TGIF, Flag-TβRI, Flag-TβRI.act, Flag-TβRImL45 and Flag-TβRImL45.act. Cell lysates were immunoprecipitated with anti-Myc and immunoblotted with anti-HA.
(**B**) Specificity of the anti-PCTA antibody. Cell extracts from MDCK (non transfected) or 293 cells transfected with 6xMyc-PCTA were immunoblotted with anti-PCTA.

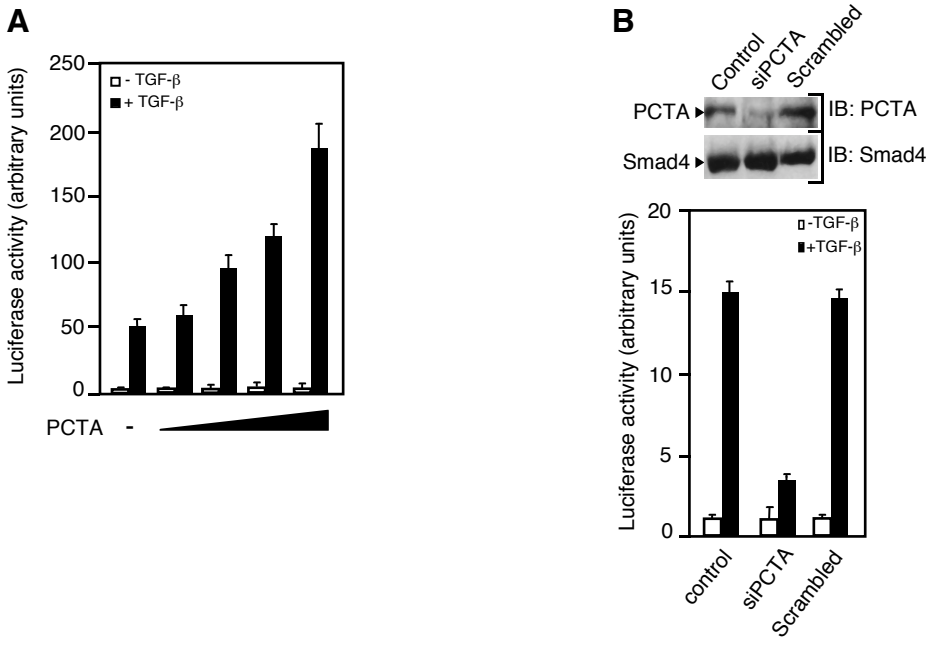


**Figure S2**

(**A**) PCTA enhances TGF-β-mediated transcriptional activation. HepG2 cells were transfected with $ARE_3$-Lux together with FAST1 and increasing amounts of PCTA. Then, cells were treated with or without TGF-β for 16 h and analyzed for luciferase activity. In this and all the following reporter assays, luciferase activity was normalized and expressed as mean ± SD of triplicates from a representative experiment performed at least three times.

(**B**) Specificity of the PCTA siRNA. 293 cells were transfected with either Scrambled or PCTA siRNA and cell extracts were analyzed by immunoblotting using anti-PCTA or anti-Smad4 as a loading control (top). 293 cells were transfected with $ARE_3$-Lux together with FAST1 and either Scrambled or PCTA siRNA. Then cells were treated with or without TGF-β for 16 h and analyzed for luciferase activity (bottom).

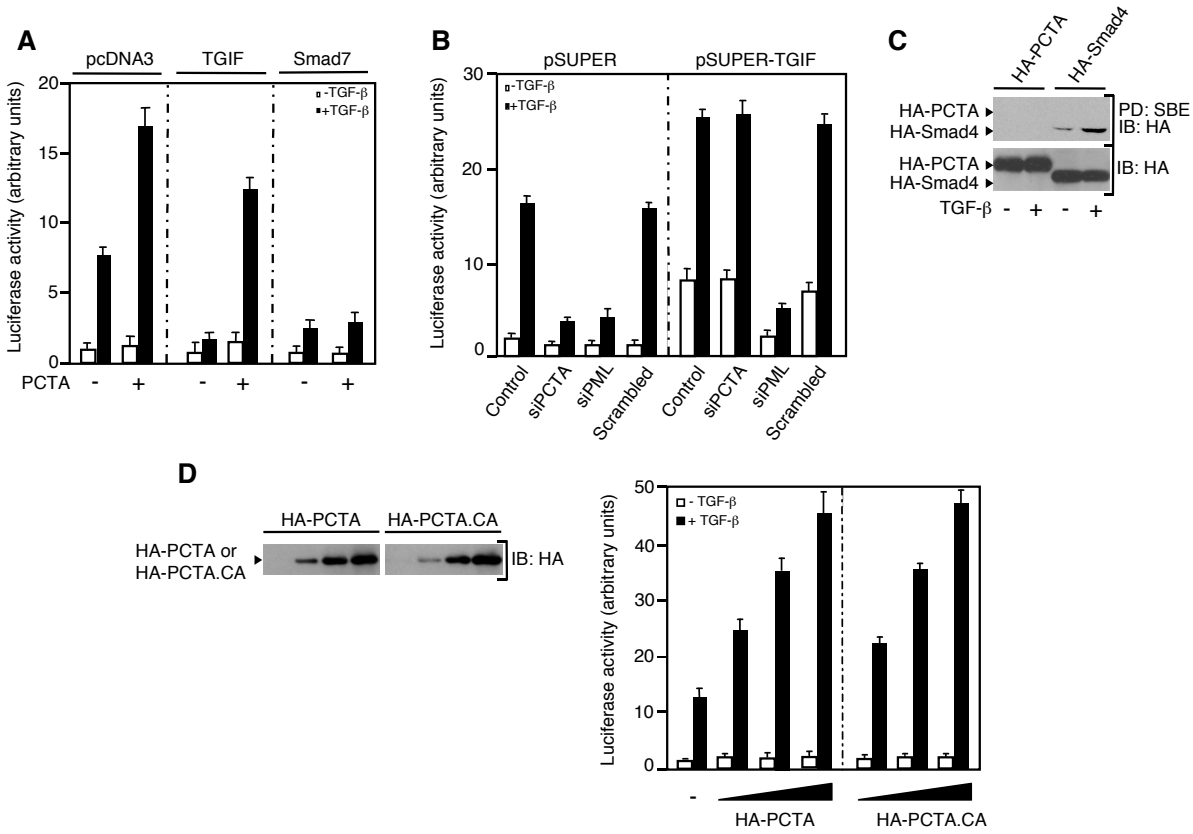


**Figure S3**

(**A**) PCTA relieves suppression of TGF-β-induced gene expression by TGIF. MDCK-pcDNA3, MDCK-TGIF or MDCK-Smad7 cells were transfected with $ARE_3$-Lux together with FAST1 in the presence or absence of PCTA. Twenty-four h later, cells were exposed to TGF-β for 16 h and analyzed for luciferase activity.

(**B**) PCTA promotes TGF-β transcriptional responses by a mechanism that is dependent on the presence of TGIF. MDCK-pSUPER or MDCK-pSUPERTGIF cells were transfected with $ARE_3$-Lux together with FAST1 and Scrambled, PCTA siRNA or PML siRNA as indicated. Then, cells were exposed to TGF-β for 16 h and analyzed for luciferase activity.

(**C**) PCTA does not function in TGF-β signaling through its ability to modulate transcription. 293 cells were transfected with HA-PCTA or HA-Smad4 and treated with or without TGF-β for 1 h. Cell extracts were pulled down with biotinylated SBE (Smad binding element) oligonucleotides and analyzed by immunoblotting with anti-HA.

(**D**) PCTA does not function in TGF-β signaling through an ubiquitin-dependent degradation mechanism. HepG2 cells were transfected with increasing amounts of wild-type HA-PCTA or HA-PCTA.CA (HA-PCTA.C503A/C506A) and the expression levels of these mutants were determined by direct immunoblotting with anti-HA (left). HepG2 cells were transfected with $ARE_3$-Lux together with FAST1 and increasing amounts of wild-type HA-PCTA or HA-PCTA.CA. Cells were treated with or without TGF-β for 16 h and analyzed for luciferase activity (right).

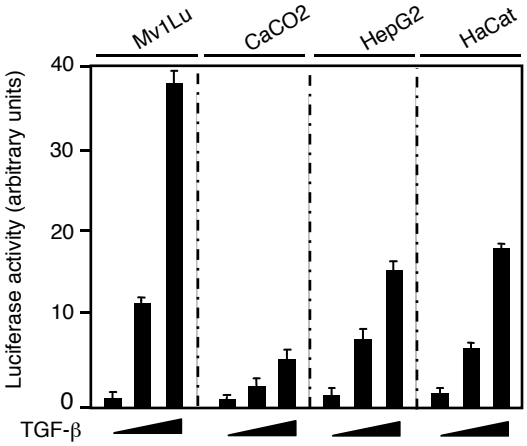


**Figure S4**
Characterization of the sensitivity of different cell lines to TGF-β-mediated transcription. Mv1Lu, CaCO2, HepG2 and HaCat cells were transfected with $ARE_3$-Lux together with FAST1. Twenty-four h after transfection, cells were treated with increasing amounts of TGF-β for 16 h and analyzed for luciferase activity.

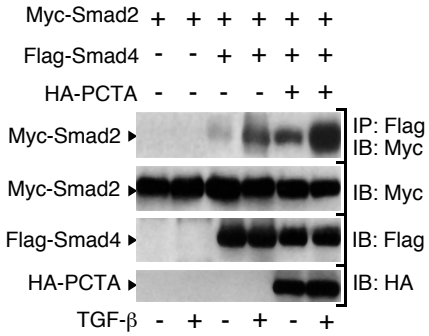


**Figure S5**
PCTA enhances the association of Smad2 with Smad4. 293 cells were transfected with Myc-Smad2 and Flag-Smad4 in the presence or absence of HA-PCTA. Forty-eight h after transfection, cells were treated with or without TGF-β for 1 h and the association of Smad2 with Smad4 was analyzed by blotting anti-Flag immunoprecipitates with anti-Myc.

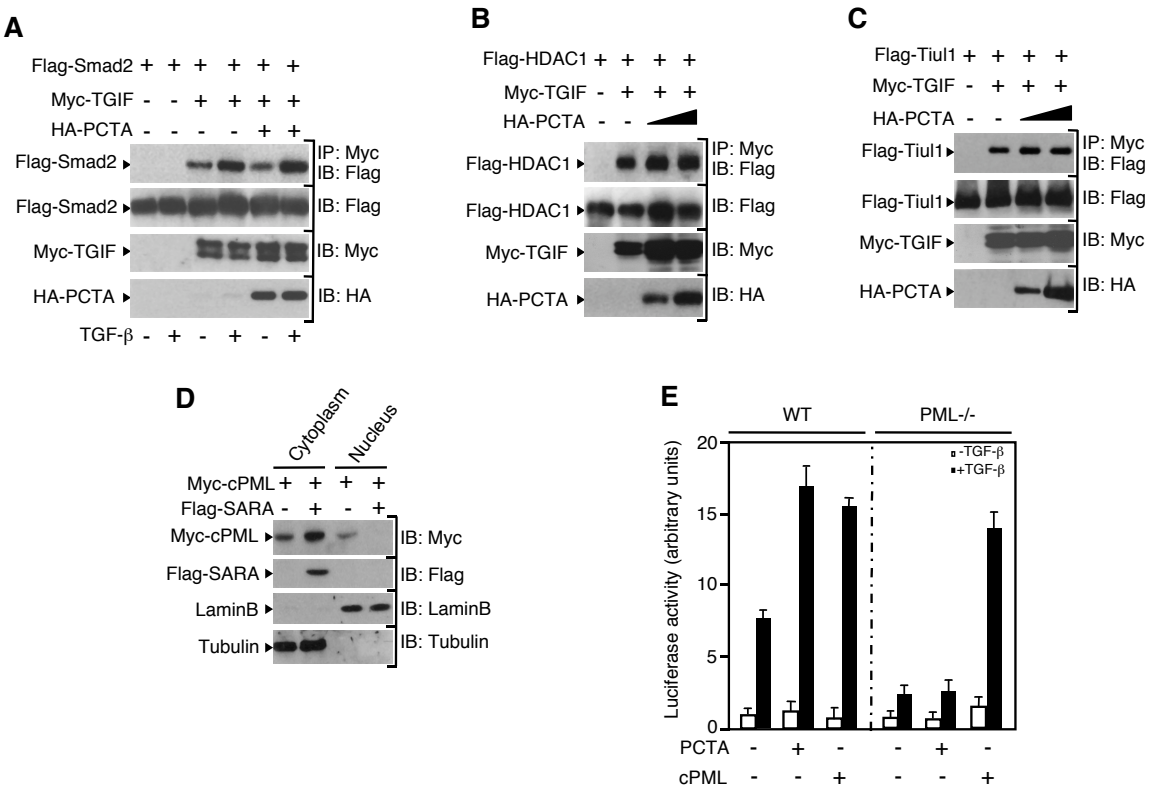


**Figure S6**

(**A**) Expression of PCTA had no effect on the association of TGIF with Smad2. 293 cells were transfected with Flag-Smad2 together with Myc-TGIF in the absence or presence of HA-PCTA. Then, cells were treated with or without TGF-β for 1 h and the association of Smad2 with TGIF was visualized by blotting anti-Myc immunoprecipitates with anti-Flag.

(**B**) Expression of PCTA had no effect on the association of TGIF with HDAC1. 293 cells were transfected with Flag-HDAC1 together with Myc-TGIF and increasing amounts of HA-PCTA. The association of HDAC1 with TGIF was visualized by blotting anti-Myc immunoprecipitates with anti-Flag.

(**C**) Expression of PCTA had no effect on the association of TGIF with Tiul1. 293 cells were transfected with Flag-Tiul1 together with Myc-TGIF and increasing amounts of HA-PCTA. The association of Tiul1 with TGIF was visualized by blotting anti-Myc immunoprecipitates with anti-Flag.

(**D**) Cytoplasmic retention of cPML by SARA. 293 cells were transfected with Myc-cPML either in the presence or absence of Flag-SARA. Then, cytoplasmic and nuclear fractions were prepared and subjected to immunoblotting analysis with anti-Myc or anti-Flag and LaminB or Tubulin as controls for the purity of fractions.

(**E**) The effect of PCTA on TGF-β-induced transcription is mediated by cPML. Wild-type or PML-/- MEFs were transfected with $ARE_3$-Lux together with FAST1 in the absence or presence of PCTA or cPML. Twenty-four h after transfection, cells were treated with or without TGF-β for 16 h and analyzed for luciferase activity.